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High prevalence of *Mycobacterium avium* subspecies *paratuberculosis* ('Indian bison type') in animal attendants suffering from gastrointestinal complaints who work with goat herds endemic for Johne's disease in India

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SUMMARY

Objectives: In this study we aimed to estimate the prevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in animal attendants who were chronic colitis patients or who had inflammatory bowel disease and were suspected for Crohn's disease; these animal attendants worked with goat herds endemic for Johne's disease. Microscopic examination and culture tests were used. For comparison purposes a group of healthy human subjects (not suffering with colitis) was also screened.

Methods: Stool samples obtained from 98 human subjects (58 animal attendants suspected for Crohn's disease and 40 healthy humans) were screened for the presence of MAP by microscopic examination and culture. Of the 58 animal attendants screened, 38 had abdominal pain, 29 had suffered episodes of diarrhea, 39 had experienced weight loss, 27 had fever, and 32 had a history of raw milk consumption. Animal attendants had had contact of variable duration with goat herds endemic for Johne's disease (1–5, 6–10, 11–15, and >15 years). Forty stool samples from healthy humans with no symptoms/history of contact with animals were also screened. IS*900* PCR and IS*1311* PCR restriction endonuclease analysis were used to characterize and genotype the MAP colonies.

Results: MAP was recovered from 34 of the 98 human subject stool samples (34.7%). Of the 98 samples, 16.3% ($n = 16$) were acid-fast. None of the 40 healthy human subjects were positive for MAP by microscopy, but five (12.5%) were positive for MAP by culture. Of the 58 animal attendants, 16 (27.6%) were positive by microscopy and 29 (50%) were positive by culture. MAP were recovered from 68.4% of animal attendants with abdominal pain, 72.4% of those with diarrhea, 71.8% of those with weight loss, 44.4% of those with fever, and 46.9% of those who had a history of raw milk consumption. Of the 29 culture-positive animal attendants, 48.3% had worked for >15 years, 27.6% for 11–15 years, 20.7% for 6–10 years, and 3.4% for 1–5 years with goat herds endemic for Johne's disease. Of the 34 culture isolates, 28 (82.4%) showed good quality DNA on agarose gel and were positive by IS*900* PCR. Of the 28 IS*900*-positive DNA samples, 23 (82.1%) were genotyped as 'Indian bison type' and five (17.9%) as 'cattle type'.

Conclusions: The prevalence of MAP was higher in attendants suffering from gastrointestinal problems who worked with goat herds endemic for Johne's disease, than in humans with no history of contact with animals. The risk of developing gastrointestinal problems with clinical symptoms indistinguishable from inflammatory bowel disease was higher in humans who were in contact with goat herds endemic for Johne's disease as compared to healthy humans, and the risk was correlated with the duration of association with the endemic goat herds.

1. Introduction

Pathogens that are transmitted between the environment, animals (domestic or wild), and humans present major challenges for animal and human health in overpopulated countries. In India, goat husbandry is a very popular means of livelihood and a regular source of income for millions of landless poor and marginal farmers. Therefore a significant proportion of the rural and urban poor keep goats for their livelihood and nutritional (milk and meat) security. India possesses the highest livestock population (485 million) worldwide, and presently ranks second in the world, after China, with respect to human population. Of the total working population, 5.5% is dependent on the livestock sector for their

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livelihood. India is the leading milk producer and ranks second for meat production in the world. However, per-animal productivity is well below the Asian and world averages.[1] A factor that contributes greatly to this reduced productivity is the presence of chronic infectious diseases that are difficult to diagnose and control. Paratuberculosis or Johne's disease (JD) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is one such chronic and incurable disease.

Johne's disease is endemic on farms and in farmer herds/flocks in every state of the country.[2] In North India, a high prevalence of JD has been found in domestic livestock using indigenous, sensitive, and MAP-specific tests.[3,4] MAP, the causative agent of JD, has been incriminated as the causative agent of Crohn's disease (CD) in humans. CD is considered a non-specific chronic inflammatory condition of the gastrointestinal tract and shares the pathology of JD in cattle. Active disease is characterized by reduced appetite, abdominal pain, bloody diarrhea, vomiting, weight loss, and tiredness.[5]

Numerous researchers have detected MAP more frequently in CD patients than in ulcerative colitis patients or controls.[6–9] Naser et al.,[7] reported that MAP infection in the human is systemic, like paratuberculosis, and cultured viable MAP from the blood of CD patients (55%) and ulcerative colitis patients (22%), but not from controls. This controversy was greatly intensified when MAP was reported to be present in human breast milk[6] and in commercial pasteurized milk,[10,11] thus showing milk to provide an important mode of transmission for this bacillus. MAP may be transmitted from animals to humans via milk, water, and other foodstuffs.[12,13] Although MAP is still considered primarily an animal pathogen worldwide, Richter et al. have reported MAP from immunocompromised patients in Germany.[14]

Close bonding between animals and the human population, the endemicity of MAP in the animal population, and increasing reports on the presence of MAP in CD patients may shed light on the association of MAP with CD. However, such data are limited and inconclusive with regard to showing a direct link between MAP and cases of colitis or inflammatory bowel disease (IBD) or CD.

The present study was based on the hypothesis that persons working with goat herds endemic for JD would be at higher risk for exposure to MAP, which may later lead to the development of colitis or IBD or CD. Therefore, goat attendants suspected for CD and working with herds endemic for JD were screened for the presence of MAP, and the association between the two (MAP and CD) was investigated.

2. Materials and methods

2.1. Collection of stool samples

A total of 88 animal attendants (animal care workers) working with goat herds endemic for JD were interviewed for the presence of a clinical profile indistinguishable from CD, ulcerative colitis, and non-specific idiopathic IBD (infrequent bowel movement, tendency to get tired easily, frequent abdominal pain, frequent episodes of diarrhea, gradual weight loss, low grade fever, etc.) and their history of raw milk consumption during the period 2005–2008. Of the 88 animal attendants, 68 (77.3%) suffered with at least one of the four symptoms of CD (diarrhea, weight loss, abdominal pain, and low grade fever) and were regarded as suspected for CD. Of the 68 animal attendants, 58 volunteered for the present study and provided stool samples. Animal attendants without symptoms of IBD ($n = 20$) did not provide any sample and also did not agree to participate in the study.

Of the 58 animal attendants included in the study, 38 had frequent abdominal pain, 29 had episodes of diarrhea, 39 had experienced weight loss, and 27 had low grade fever (the clinical symptoms of CD); 32 also had a history of raw milk consumption. One animal attendant was suffering with rectal bleeding. Animal attendants had been working with animal herds endemic for JD for variable durations (1–5, 6–10, 11–15, and >15 years). Of the 58 stool samples obtained, 40 were from individuals working with goat herds endemic for JD at the Central Institute for Research on Goats (CIRG), Makhdoom,[15–17] eight were from farmers from the town of Farah, and 10 were from the village of Jhandipur (around the CIRG campus). These workers were poor, illiterate, and could not afford the cost of medical assistance; therefore, they neither continued with treatment nor sought advance medical interventions for the diagnosis of the type of colitis (IBD). These workers also had their own stocks of goats and sheep.

Forty stool samples were collected from healthy human subjects with no history of contact with JD-infected animals. Of the 40 stool samples, 26 were from people who lived in the Mathura region, and these persons did not have clinical symptoms of IBD. The other 14 stool specimens were obtained from non-IBD patients attending Asopha Hospital and Research Center, Agra. These persons were easily convinced to volunteer for the study. Samples were collected in sterilized containers and transported to the laboratory under ice without adding any preservative, and stored at −20 °C until further use.

2.2. Processing of stool samples

Stool samples were concentrated by centrifugation and stained with Ziehl–Neelsen (ZN) stain. MAP was isolated as per the method of Whipple et al.,[18] with some modifications.[19] Briefly, approximately 2 g of stool sample was finely ground in sterilized distilled water (10–12 ml) in a sterilized pestle and mortar. The ground material was transferred to 15-ml centrifuge tubes. Tubes were centrifuged at $1557 \times g$ for 1 h at room temperature. The supernatant was discarded and the middle layer decontaminated in 25 ml of 0.9% hexadecylpyridinium chloride for 18–24 h at room temperature. After decontamination and sedimentation, supernatant was removed and discarded slowly. About 1 ml of sediment was left for the inoculation of Herrold's egg yolk medium slants (three containing mycobactin J and one without mycobactin J) and preparation of smears. Slants were incubated at 37 °C and screened for the appearance of colonies every 15 days.

2.3. IS900 PCR (colony PCR)

Cultures were processed for DNA isolation as per van Embden et al.[20] and van Soolingen et al.[21] DNA was amplified by PCR using IS*900* primers.[22] The 229-bp fragment targeting the specific IS*900* sequence was amplified from template DNA. Briefly, the following were included in a total volume of 50 μl of reaction mixture: 1 μl of each primer (forward primer: 150 C 24-mer; reverse primer: 921, 25-mer), 22 μl Red Dye Master Mix (Taq DNA polymerase, dNTPs, reaction buffer with 1.5 nM magnesium chloride), 24 μl de-ionized water, and 2 μl of template DNA. A total of 35 cycles was performed in a thermocycler (MJ Research) for a complete amplification reaction. The total time taken for 36 cycles were 1.20 h. The reaction conditions were: initial denaturation at 94 °C for 4 min (1 cycle), denaturation at 94 °C for 10 s, annealing at 61 °C for 10 s, extension at 72 °C for 10 s (35 cycles), and a final extension at 72 °C for 10 min. The presence and yield of the specific PCR product (229 bp) was analyzed by 1.8% agarose ethidium bromide gel electrophoresis. Positive (MAP 'bison type') and negative (sterilized liquipure water) controls were also run to check for contamination.

2.4. IS1311 PCR

IS*1311* PCR was carried out using M56 and M119 primers as per Sevilla et al.,[23] with some modifications. Briefly, the PCRs were set

Table 1
Screening of human stool samples for *Mycobacterium avium* subspecies *paratuberculosis* by microscopy and culture

Profile of humans	Place/region	Stool samples	Microscopy-positive (%)	Stool culture-positive (%)
Animal attendants with symptoms (suspected for CD)	CIRG	40	12 (30)	23 (57.5)
	Farah	8	2 (25)	4 (50)
	Jhandipur	10	2 (20)	2 (20)
	Total	58	16 (27.6)	29 (50)
Healthy humans	Agra	14	0	2 (14.3)
	Mathura	26	0	3 (11.5)
	Total	40	0	5 (12.5)

CD, Crohn's disease; CIRG, Central Institute for Research on Goats.

up in volume of 25 μl, using 0.5–1.0 ng template DNA, 2.5 ml of 10 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 U Taq (Promega, Madison, WI). Thermal cycling was as follows: initial denaturation at 94 °C for 3 min, followed by 37 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. An amplicon size of 608 bp was considered positive in IS*1311* PCR, after separation on a 2% agarose gel stained with ethidium bromide.

2.5. IS1311 PCR-restriction endonuclease analysis (REA)

IS*1311* PCR-REA was carried out as per Sevilla et al.[23] Briefly, the reaction was carried out in a volume of 30 μl, containing 20 μl positive IS*1311* PCR product, 3 μl 10 × buffer, and 2 U of each endonuclease HinfI and MseI (Fermentas, USA). The reaction mixture was incubated at 37 °C for 1.5 h. Band patterns were visualized after electrophoresis on a 4% agarose gel and staining with ethidium bromide. Genotype profiles were interpreted as per Whittington et al.[24]

3. Results

3.1. Microscopic examination

Screening of the 98 stool samples collected from human subjects (58 animal attendants and 40 healthy humans) by microscopy, showed the presence of acid-fast bacilli (AFB) that were morphologically indistinguishable from MAP in 13.3% (n = 16) (Table 1). These AFB were pink-colored short bacilli, usually arranged in clumps (Figure 1). Of the 58 stool samples from animal attendants suspected for CD, 27.6% (n = 16) showed the presence of AFB morphologically indistinguishable from MAP. None of the stool samples from the healthy humans was positive for MAP on microscopy. Of the 16 positive animal attendants, 75% (n = 12) were from CIRG Makhdoom, 12.5% (n = 2) from the town of Farah, and 12.5% (n = 2) from the village of Jhandipur (Table 1).

The symptom profile and association with microscopy was also studied (Table 2). Of the 58 animal attendants who were positive by microscopy (16/58, 27.6%), 81.3% (13/16) had a clinical profile of abdominal pain, 75% (12/16) diarrhea, 87.5% (14/16) weight loss, and 50% (8/16) mild fever. With regard to raw milk consumption, of the 58 animal attendants who were positive for MAP by microscopy, 43.8% (7/16) had a history of raw milk consumption. Of the 16 animal attendants positive for MAP by microscopy, 56.3% had worked >15 years, 37.5% 11–15 years, 6.3% 6–10 years, and 0% 1–5 years with goat herds endemic for JD (Table 3). The rate of MAP detection in the stool samples of animal attendants was directly proportional to the duration of association with goat herds endemic for JD. The longer the duration of association, the higher the chance of detecting AFB (Table 3).

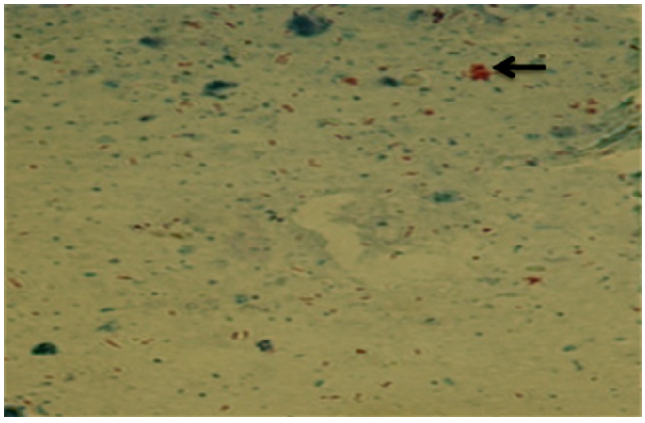

Figure 1. Ziehl–Neelsen staining showing acid-fast bacilli indistinguishable from *Mycobacterium avium* subspecies *paratuberculosis* in stool samples from animal attendants (under oil immersion microscope, ×100).

3.2. Isolation and characterization of MAP colonies

Stool samples of animal attendants suspected for CD and working with goat herds endemic for JD were screened for the presence of MAP using culture. Of the 58 stool samples, 50% (n = 29) were positive for typical MAP colonies after 180 days of inoculation (Table 1). The prevalence of MAP was higher in animal attendants at CIRG Makhdoom (57.5%) than in those from Farah town (50%) and from Jhandipur village (20%). Of the 40 stool samples from healthy humans, 12.5% (n = 5) were positive for MAP bacilli on culture. The prevalence of MAP was higher in healthy humans from Agra (14.3%) as compared to Mathura (11.5%).

Of the 58 animal attendants who were positive for MAP on culture (29/58, 50%), 89.7% (26/29) had a clinical profile of abdominal pain, 72.4% (21/29) diarrhea, 96.6% (28/29) weight

Table 2
Correlation between symptom history profile of animal attendants (n = 58) and the recovery of *Mycobacterium avium* subspecies *paratuberculosis*

Symptom history profile	No. animal attendants	Microscopy-positive (%)	Stool culture-positive (%)
Abdominal pain	38	13 (34.2)	26 (68.4)
Diarrhea	29	12 (41.4)	21 (72.4)
Weight loss	39	14 (35.9)	28 (71.8)
Mild fever	27	8 (29.6)	12 (44.4)
History of raw milk consumption	32	7 (21.9)	15 (46.9)

Table 3
Correlation between isolation of *Mycobacterium avium* subspecies *paratuberculosis* bacilli and length of association of animal attendants with goat herds endemic for Johne's disease

Duration (in years) of association with goat herds endemic for JD	No. animal attendants	Microscopy-positive (%)	Stool culture-positive (%)
1–5	4	0 (0)	1 (3.4)
6–10	12	1 (6.3)	6 (20.7)
11–15	15	6 (37.5)	8 (27.6)
>15	27	9 (56.3)	14 (48.3)
Total	58	16 (27.6)	29 (50)

JD, Johne's disease.

loss, and 41.4% (12/29) mild fever (Table 2). With regard to raw milk consumption, of the 58 animal attendants who were positive for MAP bacilli on culture, 51.7% (15/29) had a history of raw milk consumption. There was no difference in the rate of recovery of MAP (50% each) from animal attendants with and without history of raw milk consumption. Individually, 58.6% (17/29) attendants with symptoms of diarrhea, 57.9% (22/38) with abdominal pain, 48.7% (19/39) with weight loss, and 55.6% (15/27) with mild fever were consuming raw milk in their diet. A person suffering from rectal bleeding was also positive for the recovery of MAP bacilli.

Of the 29 culture-positive animal attendants, 48.3% had worked >15 years, 27.6% had worked 11–15 years, 20.7% had worked 6–10 years, and 3.4% had worked 1–5 years with goat herds endemic for JD (Table 3). The recovery of MAP isolates was greater from those animal attendants who had worked for >15 years, followed by those who had worked 11–15 years and 6–10 years. The majority of MAP isolates recovered were paucibacillary, with minute colonies in primary culture after 180 days of incubation.

3.3. Comparative detection of MAP by microscopy and stool culture

Of the 98 stool samples screened, 16.3% were positive for MAP by microscopy and 34.7% by culture (a cumulative 38.8% positive by the two tests). There was an agreement of 73.5% and a mismatch of 26.5% for the two tests; 4.1% were positive exclusively by microscopy and 22.4% were positive exclusively by culture. Cumulatively 56.9% of animal attendants and 12.5% of healthy humans were positive by microscopy and culture. There was an agreement of 63.8% and 87.5% and mismatch of 36.2% and 12.5% between the two tests in animal attendants and healthy humans, respectively. Independently, 29.3% and 12.5% samples were detected positive by culture in animal attendants and healthy humans, respectively. None of the stool samples from healthy humans were positive for MAP by microscopy; 6.9% of animal attendants were positive exclusively by microscopy (Table 4).

Table 4
Comparative detection of *Mycobacterium avium* subspecies *paratuberculosis* in stool samples from humans by microscopy and stool culture

Samples	Combinations			
	1	2	3	4
Microscopy	+	–	+	–
Stool culture	+	–	–	+
Animal attendants (n = 58)	12	25	4	17
Healthy humans (n = 40)	0	35	0	5

+, positive; –, negative. Agreement 73.5% (72/98); mismatch 26.5% (26/98).

3.4. IS900 PCR and IS1311 PCR

Of the 34 MAP cultures (29 from animal attendants and five from healthy humans) processed for DNA isolation and IS*900* PCR, 29 (85.3%) were amplified giving the specific 229-bp PCR product. Of the 29 cultures from animal attendants with a suspected infection, 82.8% (n = 24) were characterized as MAP. Of the five cultures from healthy persons, four (80%) were positive. DNA could not be isolated from the remaining samples due to the presence of few and very minute colonies of MAP on Herrold's egg yolk medium with mycobactin J.

3.5. Genotyping of MAP isolates by IS1311 PCR-REA

Of the 28 IS*900*-positive MAP isolates of human origin, 23 (82.1%) were 'Indian bison type' and five (17.9%) were 'cattle type'. Of the 24 MAP isolates from animal attendants, 22 (91.7%) were 'Indian bison type' and two (8.3%) were 'cattle type'. In animal attendants, 19 isolates were recovered from stool samples of those working with MAP endemic goat herds at CIRG Makhdoom; 18 (94.7%) were 'Indian bison type' and one (5.3%) was 'cattle type'. Of three isolates from Farah town, two (66.6%) were 'Indian bison type' and one (33.3%) was 'cattle type'. The two isolates from Jhandipur village were both 'Indian bison type' (Table 5). In healthy human subjects the 'cattle type' genotype of MAP was more prevalent and was recovered from 75% of the MAP isolates.

4. Discussion

Despite the high prevalence of JD in livestock herds and the emerging global concerns for zoonotic MAP, the status of MAP and resulting economic losses have not been estimated in the huge livestock population (177 million cattle, 98.7 million buffalo, 125.4 million goats, and 64.2 million sheep) and human population (more than 1 billion) of India. The present study investigated the

Table 5
Genotype profiles of *Mycobacterium avium* subspecies *paratuberculosis* isolates of human origin

Subject	Sample	Region	Culture	IS*900*	Genotype	
					'Indian bison type'	'Cattle type'
Animal attendants	Stool	CIRG	23	19	18	1
		Farah	4	3	2	1
		Jhandipur	2	2	2	0
		Total	29	24 (82.7%)	22 (91.7%)	2 (8.3%)
Healthy humans	Stool	Agra	2	1	0	1
		Mathura	3	3	1	2
		Total	5	4 (80%)	1 (25%)	3 (75%)

CIRG, Central Institute for Research on Goats.

prevalence of MAP in animal attendants who have symptoms consistent with CD, ulcerative colitis, and non-specific idiopathic IBD, who worked with goat herds endemic for JD, and compared these persons with healthy humans.

Of the 98 human stool samples, 58 were from animal attendants suspected for CD and 40 were from healthy humans. Microscopy showed that 27.6% of stool samples from the animal attendants were positive for typical pink staining short rods of MAP using ZN stain. However, a few studies have reported that MAP in humans is present in the cell-wall deficient (CWD or spheroplast) form.[25,26] It has been predicted that the presence of CWD forms of MAP in humans depends on the immune response of the host.[26]

These animal attendants were working with goat herds endemic for JD.[15–17] The presence of AFB in stool samples of animal attendants may be a simple pass-through phenomenon, since herds are endemic[15–17] and the MAP load in the environment/soil at CIRG, Makhdoom is heavy due to daily shedding of MAP bacilli. Therefore animal attendants may be receiving a daily dose of MAP, which may be passed through the stool without establishing an infection. However, there has been no such report; hence conclusions cannot be drawn in favor of this argument. Also, it is possible that the immune systems of the animal attendants were weakened by either infection or other reasons to such an extent that MAP colonized and multiplied in human tissues in the same way as seen in animals.[16,17] This second hypothesis is supported by a previous observation in which AFB indistinguishable from MAP were seen in the stool and tissues of a person whose immune system was severely weakened due to AIDS.[14]

Of the 40 stool samples from healthy humans, none were positive for AFB (ZN-positive). The absence of ZN-positive MAP in healthy humans may be due to the presence of the non-acid-fast staining form of the bacilli (spheroplast)[25,26] and/or visualization of smears using a microscope at ×100 magnification. Visualization of individual bacterial forms has been shown to require magnification at ×1000 in CD patients.[27,28] Previous studies have also reported the presence of the spheroplast form of MAP in the tissues of animals with JD.[29]

Isolation of MAP from clinical samples is the most definite test in the diagnosis of MAP infection.[30] However, previous studies have reported that the recovery of MAP from human subjects is particularly difficult and requires a prolonged incubation period.[25,26] The present study also showed MAP colonies of human origin to be highly fastidious and extremely slow growing – only tiny colonies were visible even after prolonged incubation. Of the 98 stool samples, 34 (34.7%) were positive on culture.

Individually, 50% of stool samples from animal attendants and 12.5% from healthy human subjects were positive. The high MAP recovery rate from animal attendants suspected of CD working with endemic goat herds may indicate that the disease has spread from goats to humans. Further it was found that close contact with infected animals may increase the risk of acquiring infection by humans. The symptom profile of animal attendants with suspected CD showed that the rate of MAP recovery increased with the increasing severity of the symptoms. In the present study it was found that clinical symptoms of diarrhea and weight loss were frequently associated with the recovery of MAP in the animal attendants (Table 2). In animal attendants, diarrhea was chronic, frequent (more than four times/day), and more watery, like IBD patients. The person with bloody diarrhea was positive for the presence of MAP. This finding indicates that MAP may be consistent with an infectious cause of these conditions.

However, the present study does not confirm that diarrhea and weight loss were exclusively due to the MAP infection in animal attendants. Amebiasis and typhoid are the most common of health problems in India and these people are very poor and seldom obtain a differential diagnosis. Awareness of CD and cases of CD have been on the increase in India, and patients are very touchy about their data being shared.

In the present study, there was no difference in the rate of recovery of MAP from animal attendants with and without history of raw milk consumption. These findings indicate that the consumption of raw milk was not a major source of MAP infection in the animal attendants as compared to fecal–oral transmission (from contact with infected manure during cleaning of sheds and paddocks), drinking of treated or untreated contaminated water, and inhalation of water droplets (aerosols) contaminated with MAP at the goat farm (endemic for JD). Pickup et al.,[31] have also reported inhalation of water droplets as a transmission route for MAP to humans.

The correlation between isolation of MAP and the duration of association of animal attendants with endemic JD herds was also investigated. The highest percentage of MAP-positive animal attendants had worked for more than 15 years in the JD-infected herds, followed by those who had worked 11–15, 6–10, and 1–5 years (Table 3). These findings indicated that the risk of acquiring gastrointestinal problems indistinguishable from IBD is increased with the rate and duration of association with clinical MAP-infected animals. Similar to the present study, Jones et al.,[32] reported that frequency of contact with clinical cases of bovine paratuberculosis confers an eight-times increased risk of ulcerative colitis in humans. The presence of MAP in the stool of a few healthy humans (12.5%) could be attributed to environmental contamination or to exposure that may occur through the food chain or water supply.[33,34]

For molecular characterization of MAP isolates, specific IS*900* PCR (specific and nested) was employed. However, some studies have reported the presence of similar sequences in other mycobacterial isolates: *Mycobacterium sp* strain 2333,[35] *Mycobacterium sp* strain 28850, *Mycobacterium sp* strain WA-1, and *Mycobacterium sp* strain WA-2.[36] In the present study, the presence of these rare elements was excluded using IS*1311* PCR-REA. All of the 34 culture isolates (29 from animal attendants and five from healthy humans) were subjected to DNA isolation using the physical, chemical, and enzymatic method.[20,21] Of the 34 culture isolates, 28 (82.4%) samples yielded good quality DNA. This less than 100% recovery of DNA from culture isolates may be due to the presence of extremely minute and paucibacillary colonies on culture medium. The isolation of DNA from MAP is considerably more difficult than from other bacteria in general. Previous studies have also reported a low recovery of DNA from paucibacillary forms of colonies of MAP using the same DNA isolation protocol.[37]

Genotypes of the MAP isolates were studied by IS*1311* PCR-REA method. IS*1311* PCR-REA of IS*900*-positive samples provided the advantage of both identification and genotyping of MAP. To study the predominant genotype of MAP in animal attendants and healthy humans, IS*900*-positive MAP DNA (24 from animal attendants and four from healthy humans) was subjected to genotyping; 82.1% were 'Indian bison type' and 17.9% were 'cattle type'. Previous studies from this region have also reported a high prevalence of MAP 'Indian bison type' in domestic livestock and humans.[17,38–40] Contrary to the present findings, 'cattle type' is the major genotype infecting livestock and the wild animal population worldwide.[41–43] 'Indian bison-type' MAP has not been reported so far from livestock outside India. Of the 24 MAP DNA from animal attendants suspected for CD, 91.7% were characterized as 'Indian bison type' and 8.3% as 'cattle type' (Table 5). However, in healthy human subjects, 'cattle type' was the predominant genotype, recovered from 75% of MAP isolates. The present study showed that the genotype profile in humans was the same as that seen in animals for that particular region. These findings suggest that humans acquired MAP bacilli from goats and other animals located in this area.

In conclusion, higher presence of MAP in animal attendants suffering with gastrointestinal problems and suspected for CD as compared to healthy humans indicated association between the two (MAP and CD). MAP appeared to be not only a zoonosis, but an easily transmitted one, such that humans readily become infected with and develop disease caused by MAP. Humans working with JD-infected goat herds were at higher risk of acquiring MAP through the fecal–oral transmission route. Rather than raw milk consumption, fecal–oral transmission was the major route for the transmission of MAP from animals to humans. Further, as the duration of association of a person with JD-infected animals increased, the risk of acquiring MAP and gastrointestinal problems indistinguishable from IBD may also increase. Hence, precautionary measures must be taken while handling JD-infected animals.

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